Multiple Molecular Markers as Predictors of Colorectal Cancer in Patients with Normal Perioperative Serum Carcinoembryonic Antigen Levels

Jaw-Yuan Wang,[1,2] Shiu-Ru Lin,[3] Deng-Chyang Wu,[2] Chien-Yu Lu,[2] Fang-Jung Yu,[2] Jan-Sing Hsieh,[1,2] Tian-Lu Cheng,[4] Lok-Beng Koay,[5] and Yih-Huei Uen[6]

Abstract

Purpose: In this study, a high-sensitivity colorimetric membrane array method was used to detect circulating tumor cells (CTC) in the peripheral blood of colorectal cancer (CRC) patients with normal perioperative serum carcinoembryonic antigen (CEA) levels. This membrane array method was evaluated as a potential diagnostic and postoperative surveillance tool.

Study Design: Membrane arrays consisting of a panel of mRNA markers that include human telomerase reverse transcriptase, cytokeratin-19, cytokeratin-20, and CEA mRNA were used to detect CTCs in the peripheral blood of 157 postoperative CRC patients with normal perioperative serum CEA levels and in 80 healthy individuals. Digoxigenin-labeled cDNA were amplified by reverse transcription-PCR from the peripheral blood samples, which were then hybridized to the membrane array. The sensitivity, specificity, and accuracy of membrane arrays for the detection of CTCs were then calculated.

Results: Using the four markers in combination, expression of any three markers or all the four markers in this panel was significantly correlated with the clinicopathologic characteristics, including depth of tumor invasion, lymph node metastasis, tumor-node-metastasis stage, and postoperative relapse (all $P < 0.05$). The interval between the detection of all four positive molecular markers and subsequent elevated CEA ranged from 3 to 8 months (median 6 months). The expression of all four mRNA markers was an independent predictor for postoperative relapse. CRC patients with all four mRNA markers expression showed a significantly poorer survival rate than those with less than four positive markers.

Conclusions: The constructed membrane array method was helpful in the early prediction of postoperative relapse in CRC patients with normal perioperative serum CEA levels.

Relapses have an important meaning in relation to survival for curative surgical intervention of cancer patients. Since its discovery by Gold and Freedman in 1965 (1), serum carcinoembryonic antigen (CEA) has been used extensively in clinical practice for tumor follow-up to detect recurrence in colorectal cancer (CRC) patients. An elevated preoperative CEA is a poor prognostic sign and correlates with a reduced overall survival after surgical resection. Many studies have established the usefulness of serum CEA-oriented serial monitoring for predicting recurrence and prognosis of CRC (2–7). However, few studies have thus far investigated the surveillance of patients with normal perioperative serum CEA levels.

Because early detection is an important factor in reducing cancer mortality, the development of a sensitive, specific, and convenient diagnostic method for detecting CRC at a very early stage is an issue of utmost importance, especially for CRC patients with normal perioperative serum CEA levels. Models of metastasis indicate that primary tumor cells spread to other organs via circulation (blood and lymphatics). Sensitive detection of occult carcinoma cells in the peripheral blood of CRC patients has important prognostic and therapeutic implications. Such neoplastic cells may be present in the bloodstream in very low numbers and would hardly be detected by conventional methods. With recent developments in molecular technology, the use of PCR, reverse transcription-PCR (RT-PCR), or real-time quantitative-PCR (Q-PCR) assays now permit sensitive detection of circulating tumor cells (CTC) in peripheral blood.

Accumulated reports have described the use of human telomerase reverse transcriptase (hTERT), cytokeratin-19 (CK-19), cytokeratin-20 (CK-20), and CEA mRNA as biomarkers for the detection of occult residual disease for CRC (8–13). Our recent investigations also indicate that CEA mRNA for detection

Authors' Affiliations: [1]Department of Surgery, Kaohsiung Medical University Hospital; [2]Faculty of Medicine, College of Medicine, [3]Graduate Institute of Medical Genetics, and [4]Faculty of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan; and [5]Department of Internal Medicine and [6]Division of General Surgery, Department of Surgery, Chi Mei Foundation Medical Center, Taipei Medical University, Taipei, Taiwan

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Requests for reprints: Yih-Huei Uen, Chi Mei Foundation Medical Center, 901 Chung-Hua Road, Yung-Kang City, Tainan 710, Taiwan. Phone: 886-6-2812811; Fax: 886-6-2828928; E-mail: cy61412@ms14.hinet.net and cmh7500@chimei.org.tw.

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Table 1. Characteristics of colorectal cancer patients

	No. of patients*
Mean age, y (range)	65.8 (20-91)
≧60/<60	108/49
Gender	
Male/female	78/79
Maximum tumor size (cm)	
≧5/<5	75/82
Location	
Colon/rectum	112/45
Differentiation	
Well/moderately/poorly	21/112/24
Depth of tumor invasion	
T_1+T_2 / T_3+T_4	32/125
Lymph node metastasis	
Present/absent	72/85
TNM stage	
I/II/II	15/70/72
Postoperative relapse	
Present/absent	53/104

*Unless otherwise indicated.

of CTCs in peripheral blood using RT-PCR is a rational approach for the surveillance of postoperative CRC patients (14). In addition, our developed membrane array–based multimarker assay can detect CTCs in the peripheral blood of various cancer patients (15–19). Thus, the aim of this study was to analyze CRC patients with normal perioperative serum CEA levels by a panel of molecular markers using a constructed membrane array method and evaluate their role in postoperative surveillance.

Materials and Methods

Patients. Two hundred and eighty-nine stage I to III CRC patients were admitted to the Department of Surgery of Kaohsiung Medical University Hospital for elective surgery between May 2002 and June 2004. Of these, 157 (54.3%) with perioperative serum CEA levels <5 ng/mL were enrolled in the present study. Seventy-eight patients were males and 79 were females. The mean age was 65.8 years (range 20-91 years).

All of the 157 patients underwent radical resection for the primary lesion. Preoperative serum CEA was sampled within 1 week before surgery and postoperative serum CEA was obtained at least 4 weeks later. Simultaneously, an additional 4-mL sample of peripheral blood was obtained from each CRC patient postoperatively for total RNA isolation. Peripheral blood samples were taken from 80 healthy individuals to serve as controls. To prevent contamination of epithelial cells, the blood samples were obtained through a catheter inserted into a peripheral vessel, and the first 5 mL of blood were discarded. Written informed consent was obtained from each subjects and/or guardian. Sample acquisition and subsequent use were also approved by the hospital institutional review board.

The clinical stage and pathologic features of the primary tumors were defined according to the sixth edition of the tumor-node-metastasis (TNM) staging system of the International Union Against Cancer (20). As to the histologic types, 21 were well-differentiated, 112 were moderately differentiated, and 24 were poorly differentiated carcinoma. Of the 157 CRC patients, 15 were subsequently diagnosed with stage I, 70 with stage II, and 72 with stage III. The clinicopathologic characteristics of these patients were listed in Table 1.

mRNA isolation and first-strand cDNA synthesis. Total RNA was extracted from the fresh whole blood of CRC patients and healthy volunteers using a QIAamp RNA Blood Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically on the basis of absorbance at 260 nm.

Membrane arrays. The procedure for the design and preparation of the membrane array was in accordance with our recent work (16). Oligonucleotide probes were then synthesized according to the design sequences, purified, and controlled before being grafted onto the substrates (Table 2). The newly synthesized oligonucleotide fragments were then dissolved in di-water to a concentration of 20 mmol/L, applied to a BioJet Plus 3000 nanoliter dispense system (BioDot, Inc., Irvine, CA), which blotted sequentially the four target DNAs, one housekeeping gene (*β-actin*), and one tuberculosis gene (50 nL per spot and 1.5 mm between spots) on a Nytran SuperCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) in triplicate, and then cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Each spot contained 20 ng of PCR-amplified DNA derived from sequence-verified cDNA clones. DMSO was also dispensed onto the membrane as a blank control.

Digoxigenin-labeled cDNA targets and hybridization. Four hundred nanograms of first-strand cDNA targeted for hybridization were made by reverse transcription of mRNA from the peripheral blood of the study subjects and control in the presence of digoxigenin-labeled UTP (Roche Diagnostics GmbH, Penzberg, Germany) using SuperScript II reverse transcriptase (Life Technologies Inc., Rockville, MD). The lifts were covered with ExpressHyb Hybridization Solution (BD Biosciences, Palo Alto, CA) containing DIG-11-UTP–labeled cDNA probes, and then incubated with antidigoxigenin alkaline phosphatase–conjugated antibody (Roche Diagnostics). The arrays were then incubated for hybridization at 42°C for 6 h in a humid chamber.

For signal detection, the membranes were incubated for 15 min in a chromogen solution containing nitroblue tetrazolium and 5-bromo-4-chloro3-indolyl-phosphate. The hybridized membrane arrays were then scanned using an Epson Perfection 1670 flat bed scanner (SElKO EPSON Corp., Nagano-ken, Japan). Subsequent quantification analysis of the intensity of each spot was carried out using AlphaEaseFC software (Alpha Innotech Corp., San Leandro, CA). The density ratio of each mRNA marker was divided with β-actin mRNA as an internal control. The spots that consistently displayed signal intensity higher than the cutoff value calculated from the receiver-operating characteristic (ROC)

Table 2. Oligonucleotide probe sequences of mRNA markers

mRNA marker	Sequence of probe	Length (bp)
hTERT	AAAGGTGTGCCCTGTACACAGGCGAGGACCCTGCACCTGGAT	42
CEA	CATGAGAGTCCAGGCTGTCTGAGTCAGCACAGTAAGAAAGTCCTTTCTGCTTTAA	55
CK-19	CAACAATTTGTCTGCCTCCAAGGTCCTCTGAGGCAGCAGGCTCTGG	46
CK-20	TCCGCATCTCCAACTCCAGACACACGGTGAACTATGGGAGCGATCTCACA	50
β-Actin	TCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACGCCAACACAGTGCTGTC	55

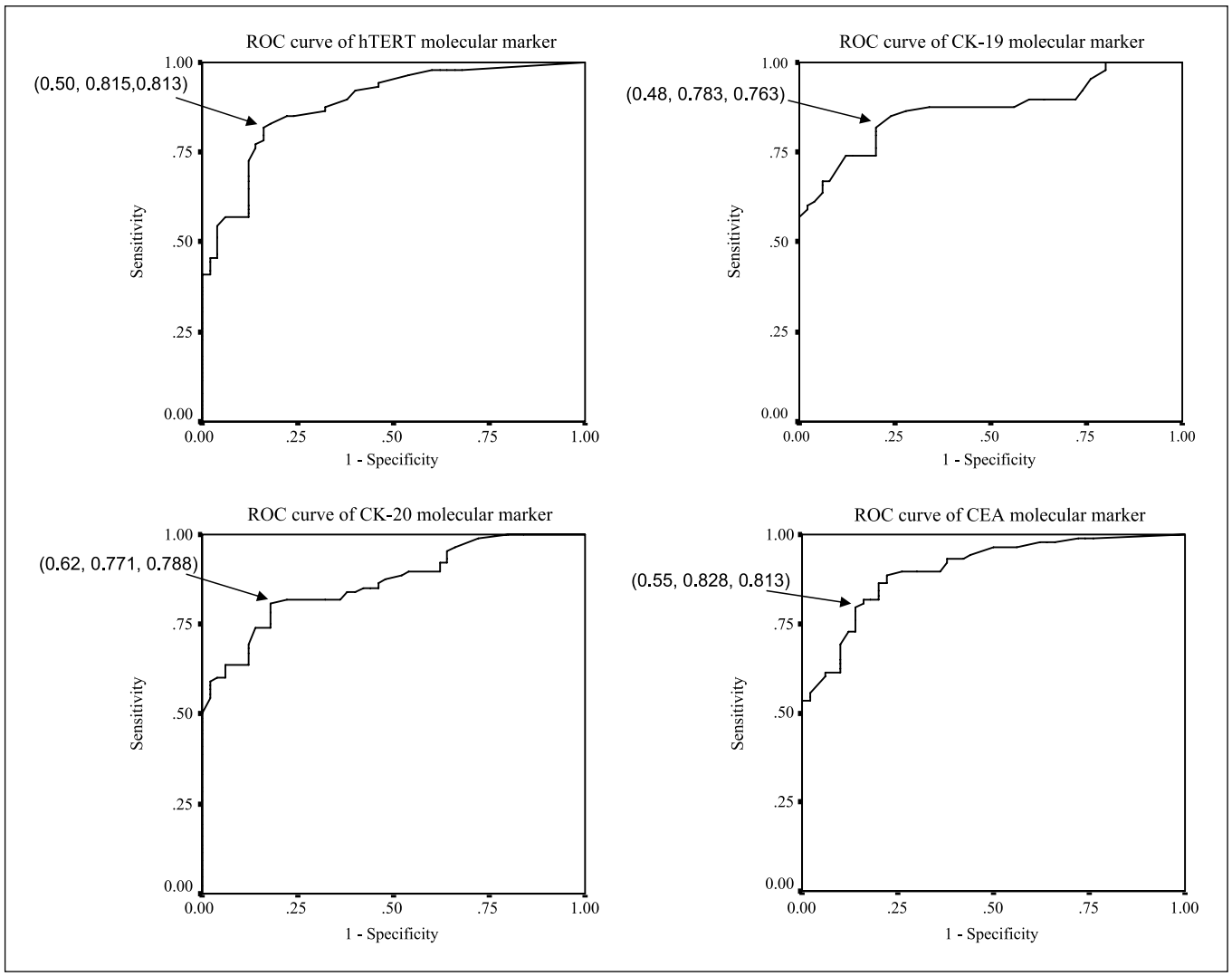

Fig. 1. ROC curves for hTERT, CK-19, CK-20, and CEA mRNA from the analysis of membrane array data of 237 subjects. Sensitivity (*Y axis*) was plotted against the false-positive fraction (*1-specificity*; *X axis*) for various cutoff values. The plot is highlighted with the numbers in parenthesis that indicate the cutoff value, sensitivity, and specificity. The area under the ROC curve for hTERT, CK-19, CK-20, and CEA mRNA is 0.885 (95% CI, 0.829-0.941), 0.868 (95% CI, 0.809-0.927), 0.866 (95% CI, 0.808-0.924), and 0.903 (95% CI, 0.853-0.952), respectively.

curves by at least one factor were considered to be positive or overexpressed.

Diagnostic accuracy of the membrane arrays. The sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy of the membrane array for CRC were analyzed. Investigators were blinded to the groups of blood samples.

Detection of serum CEA. Serum CEA levels were determined by an enzyme immunoassay test kit (DPC Diagnostic Product Co., Los Angeles, CA) with the upper limit of normal as 5 ng/mL according to the manufacturer's instructions.

Follow-up. All of the patients were carefully and regularly followed-up until their death or until June 2006 (median follow-up, 36 months;

Table 3. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of each mRNA marker and the combination between CRC patients and healthy individuals

	Sensitivity (%)	Specificity (%)	Positive predictive value (%; 95% CI)	Negative predictive value (%)	Accuracy (%)
hTERT mRNA	80.3 (66.7-93.8)	80.0 (66.4-93.6)	88.7 (77.9-99.5)	67.4 (51.4-83.4)	80.2
CK-19 mRNA	79.6 (64.0-92.2)	78.8 (64.8-92.7)	88.0 (77.0-99.1)	66.3 (50.2-82.4)	79.3
CK-20 mRNA	77.1 (62.7-91.4)	77.5 (63.3-91.7)	87.1 (75.6-98.5)	63.3 (46.8-79.7)	77.2
CEA mRNA	82.8 (69.9-95.7)	81.3 (67.9-94.6)	89.7 (79.3-100.0)	70.7 (55.1-86.2)	82.3
Any one mRNA	92.4 (83.3-101.4)	67.5 (51.5-83.5)	84.8 (72.5-97.0)	81.8 (68.7-95.0)	84.0
All four mRNA	57.3 (40.4-74.2)	100.0	100.0	54.4 (37.4-71.4)	71.7

Table 4. One hundred and fifty-seven colorectal cancer patients with or without each mRNA expression stratified according to TNM stage

	hTERT		*P*	CK-19		*P*	CK-20		*P*	CEA		*P*
	+	–		+	–		+	–		+	–	
No.	126	31		124	33		121	36		130	27	
TNM stage												
I	7	8		7	8		6	9		6	9	
II	57	13		58	12		57	13		59	11	
III	62	10	0.002	59	13	0.005	58	14	0.002	65	7	<0.0001

range, 24-49 months). At each monthly visit, physical examinations, routine blood work, serum CEA measurement, and liver function tests were conducted where appropriate. Abdominal ultrasonography was done every 6 months, as well as annual computed tomography studies for the chest and abdomen. The development of new or recurrent metastatic lesions after surgery is defined as a postoperative relapse. During the follow-up period, 53 (33.8%) patients suffered from local recurrence or distant metastasis and 38 (24.2%) expired.

Statistical analysis. All of the data were analyzed using the Statistical Package for the Social Sciences version 12.0 software (SPSS, Inc., Chicago, IL). Data were presented as mean ± SD. ROC curve analyses were done to analyze the membrane array data of hTERT, CK-19, CK-20, and CEA gene expression in the peripheral blood of the subjects. The area under ROC curve and the corresponding 95% confidence intervals (95% CI) were calculated for each marker. The cutoff value at the highest accuracy (minimal false-negative and false-positive results) was determined.

Two-sided Pearson χ^2 test and the Fisher exact test were used to analyze the potential correlation between the expression of molecular markers used in combination and the clinicopathologic features of the study subjects. To clarify the clinical significance of these mRNA markers combined into a diagnostic panel as the predictor of postoperative relapse, a multivariate adjustment was done by logistic regression analysis. The cumulative overall survival rates were calculated by the Kaplan-Meier method, and the differences in survival rates were analyzed by log-rank test.

To minimize the interpretation bias, the overall survival analysis was applied. A P value <0.05 was considered statistically significant.

Results

ROC curve analyses on the membrane array data of the 237 subjects (157 CRC patients and 80 normal individuals) were done. The ROC curves for each mRNA marker are shown in Fig. 1. Accordingly, the optimal cutoff value and area under ROC curve for each particular single mRNA marker was as follows: 0.50 and 0.885 (95% CI, 0.829-0.941) for hTERT, 0.48 and 0.868 (95% CI, 0.809-0.927) for CK-19, 0.62, and 0.866 (95% CI, 0.808-0.924) for CK-20, and 0.55 and 0.903 (95% CI, 0.853-0.952) for CEA.

The examination of single markers with membrane array displayed degrees of sensitivity, specificity, and diagnostic

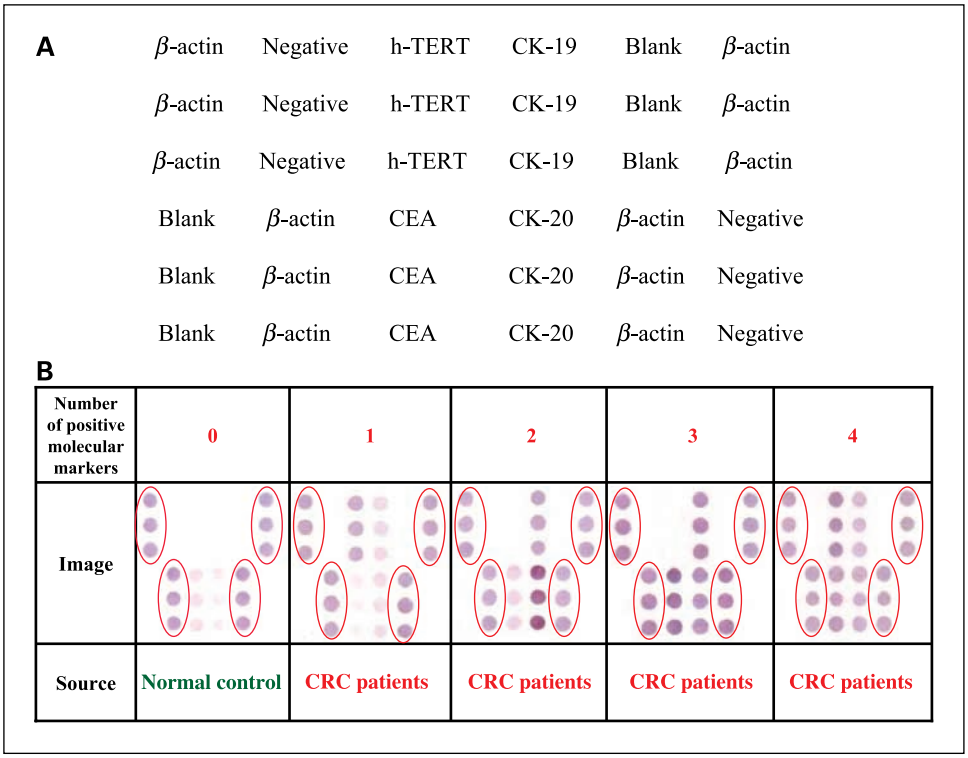

Fig. 2. *A,* schematic representation of the membrane array with four target genes, one housekeeping gene (*β-actin*), one tuberculosis gene (negative plant), and one blank control. A triplicate set of four mRNA markers for CRC was blotted on the nylon membrane. In addition, a housekeeping gene and a tuberculosis gene that served as positive and negative controls were also blotted on the membrane. *B,* comparing gene expression patterns between CRC patients and a healthy control. Spots within the red circle, β-actin (positive control). The overexpression of one to four markers was observed in four individual CRC patients, respectively, but not in the healthy individual.

Table 5. Correlation between clinicopathologic features of colorectal cancer patients and detection of four mRNA markers in combination

	Any one marker		P	Any two markers		P	Any three markers		P	All four markers		P
	+	–		+	–		+	–		+	–	
No.	151	6		140	17		128	29		90	67	
Age (y)												
≧60	104	4		96	12		87	21		66	42	
<60	47	2	0.909	44	5	0.865	41	8	0.641	24	25	0.154
Gender												
Male	75	3		68	10		65	13		44	34	
Female	76	3	0.987	72	7	0.425	63	16	0.563	46	33	0.818
Maximum tumor size (cm)												
≧5	71	4		64	11		59	16		42	33	
<5	80	2	0.345	76	6	0.139	69	13	0.377	48	34	0.748
Location												
Colon	108	4		101	11		92	20		63	49	
Rectum	43	2	0.796	39	6	0.552	36	9	0.707	27	18	0.668
Differentiation												
Well	20	1		18	3		15	6		14	7	
Moderately	108	4		102	10		96	16		60	52	
Poorly	23	1	0.9621	20	4	0.466	17	7	0.103	16	8	0.325
Depth of tumor invasion												
T_1+T_2	29	3		25	7		20	12		9	23	
T_3+T_4	122	3	0.066	115	10	0.024	108	17	0.002	81	44	<0.001
Lymph node metastasis												
Present	70	2		68	4		65	7		62	10	
Absent	81	4	0.885	72	13	0.050	63	22	0.009	28	57	<0.001
TNM stage												
I	14	1		13	2		8	7		2	13	
II	68	2		65	5		60	10		44	36	
III	69	3	0.767	62	10	0.411	60	12	<0.001	54	18	<0.001
Postoperative relapse												
Present	50	3		49	4		48	5		45	8	
Absent	101	3	0.391	91	13	0.345	80	24	0.037	45	59	<0.001

accuracy ranging from 77.1% to 82.8%, 77.5% to 81.3%, and 77.2% to 82.3% (Table 3), respectively. The false-positive rates of hTERT, CK-19, CK-20, and CEA mRNA for healthy individuals were 20%, 21.2%, 22.5%, and 18.7%, respectively. When combined with all four mRNA markers, the false-positive rate was reduced to 0%.

Table 4 shows the correlation of the sensitivity of each molecular marker in CRC patients with various TNM stages. Sensitivity was associated with advanced stages (all $P < 0.05$). Figure 2 shows the schematic representation (Fig. 2A) and representative results (Fig. 2B) of membrane array hybridization for four CRC patients and a normal control. Significant overexpression of one to four markers was observed in the peripheral blood of the four CRC patients, respectively, but not in the control sample.

As analyzed in combining these four markers with member arrays, expression of any three markers or all of the four in this panel was more significantly correlated with the clinicopathologic characteristics than any single or two markers (Table 5), including depth of tumor invasion ($P = 0.002$ and $P < 0.001$), lymph node metastasis ($P = 0.009$ and $P < 0.001$), TNM stage (both $P < 0.001$), and postoperative relapse ($P = 0.037$ and $P < 0.001$). Forty-five (50%) of the 90 patients with an overexpression of all four molecular markers and 8 (11.9%) of the 67 patients without overexpression of any molecular marker had postoperative relapse. Among the 53 patients with postoperative relapse, 45 (84.9%) overexpressed all four molecular markers.

To find out the lead time among all of the four positive molecular markers and elevated serum CEA levels (>5 ng/mL), we further analyzed 90 CRC patients with all four positive molecular markers. Of the 90, 45 developed postoperative relapse whereas 40 had elevated serum CEA levels in subsequent follow-up period (data not shown). Thirty-seven of the 40 patients with persistently elevated serum CEA levels developed postoperative relapse, whereas eight postoperative relapse patients still had normal serum CEA levels (data not shown). The interval between the 40 patients with elevated CEA levels and the presence of all four positive molecular markers ranged from 3 to 8 months (median, 6 months; Fig. 3).

The predictive values of the mRNA molecular markers or clincopathologic features for postoperative relapse were analyzed using multivariate logistic regression analysis (Table 6). The CRC patients with all four markers had a relative risk of 18.749 of developing postoperative relapse compared with those without any of the markers ($P = 0.019$). Furthermore, statistically significant difference was observed in terms of survival rate between CRC patients with expression of all four markers and those with less than four positive markers using the log-rank test (Fig. 4; $P = 0.006$).

Discussion

The only method of treatment that offers a favorable prognosis for CRC is radical resection of a part of the colon

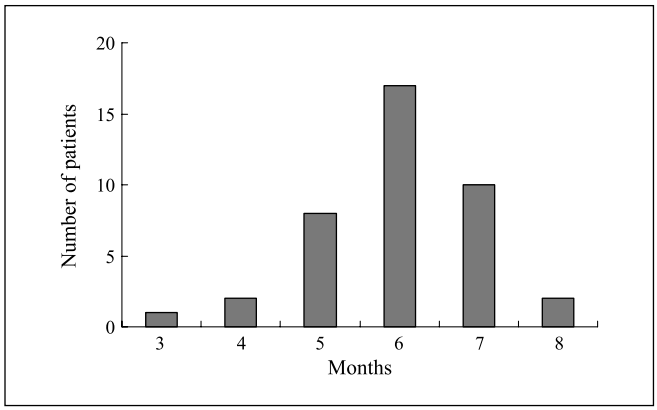

Fig. 3. The interval between subsequently elevated CEA levels and the presence of all four positive molecular markers in the 40 CRC patients.

or rectum, including the pertinent lymph glands, and a radical removal of metastases. However, even such presumably curative surgery does not ensure full recovery, as recurrences are frequent and, according to several analyses, the 5-year survival rate is <50%. The most significant reason for this poor therapeutic success is residual micrometastases.

CEA has been the most extensively investigated tumor marker for CRC. In fact, serum CEA protein is currently the most widely used marker in the surveillance or follow-up of CRC. In addition, patients with preoperative CEA levels higher than 5 ng/mL have been regarded as a high-risk group for recurrence after curative resection (5). Again, an initially elevated CEA level that fails to fall to within normal limits in the early postoperative period after curative resection may signify an additional relevant risk factor (3, 21). Nevertheless, the monitoring of recurrence or metastasis in asymptomatic patients without accompanying serum CEA elevation after curative resection has rarely been addressed.

McCall et al. (22) indicated that the diagnosis of recurrent disease for CRC patients may be made several months earlier by investigating the first abnormal CEA level. In the current investigation, we showed that our constructed colorimetric membrane array could detect CTCs in peripheral blood of CRC patients with normal perioperative serum CEA levels at an earlier stage, with a median time of 6 months earlier than the elevation of serum CEA levels. Therefore, these molecular markers might have an advantage in detecting postoperative relapse earlier for patients with normal perioperative serum CEA levels.

Indeed, 6 months is a good lead time for the introduction of new therapeutic strategies to possibly cure these patients. On the other hand, the panel of overexpressed molecular markers is prominently correlated not only to the clinicopathologic characteristics but also to postoperative relapse and overall survival rates, particularly in patients who express with all four molecular markers. These data suggest that the molecular markers have the clinical potential to be important prognostic factors for CRC patients with normal perioperative serum CEA levels after radical resection.

Likewise, Ishikawa et al. (23) has disclosed that tumor angiogenesis could predict recurrence in rectal cancer patients with normal serum CEA levels. Consistent with the findings of the angiogenesis marker from Ishikawa's group, we also observed that our multimarker panel is superior to conventional pathologic variables, such as tumor depth and lymph node involvement, for the prediction of postoperative relapse. Accordingly, concomitant molecular detection of CTCs with a multimarker panel is a justifiable supplementary approach to the current pathology staging system, which may help physicians make accurate judgment on clinical treatment and in predicting prognosis for CRC patients. A more intensive follow-up plan may be recommended for these patients despite normal perioperative serum CEA levels after radical resection.

Recently, using an RT-PCR assay for the detection of CEA mRNA has been shown to be feasible and promising as a tool for the early detection of micrometastatic CTCs (14). A multimarker panel is now the trend to enhance the sensitivity of methods for CTC detection, compared with using a single marker (24–26). The present study shows that detection sensitivity (77.1-82.8%) of the colorimetric membrane array method is considerably higher than that of our previous RT-PCR assay (52.8-72.2%) for the detection of CTCs in each corresponding molecular marker (14). This membrane array assay is more accurate in discriminating CRC patients and normal subjects.

Several recent RNA-based approaches focused only on the clinical significance of single-marker analysis. Although these methods provide useful data, one of the limitations was that the methodology could analyze only one molecular target at a time. Due to the heterogeneity of the expression of tumor-related genes, a multimarker assay is regarded as more reliable and sensitive than a single-marker assay (24–27). As expected, the detection rate and diagnostic accuracy for CTCs of CRC patients were significantly increased, as a panel of multiple markers was used, compared with single markers. Our highly

Table 6. Association between postoperative relapse and combinations of mRNA molecular markers or clincopathologic features from colorectal cancer patients using multivariate logistic regression analysis

Variables	Coefficient	SE	*P*	Hazard ratio (95% CI)
Depth (T_3+T_4 / T_1+T_2)	2.123	1.156	0.042	7.643 (1.256-67.356)
Lymph node metastasis				
Presence/absence	0.572	1.342	0.231	3.546 (0.271-16.088)
Stage (III / I+II)	0.841	0.603	0.153	2.048 (0.122-7.842)
Any one marker	−0.057	12.485	0.999	1.246 (0.362-3.259)
Any two markers	0.128	16.035	1.000	1.207 (0.657-4.253)
Any three markers	0.342	5.612	0.095	3.185 (0.453-13.464)
All four markers	2.917	1.245	0.019	18.479 (5.612-112.878)

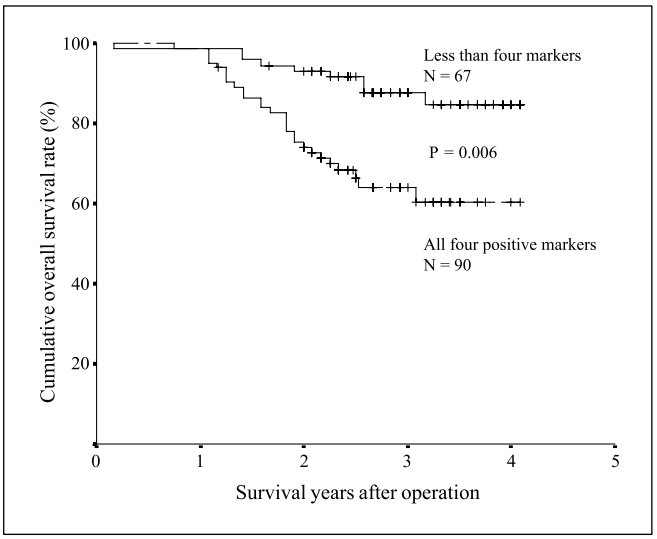

Fig. 4. Cumulative overall survival rate of 157 CRC patients by Kaplan-Meier analysis. Patients with all four mRNA markers expression in the peripheral blood showed a significantly poorer survival rate than those with less than four positive mRNA maker expression ($P = 0.006$).

sensitive and high-throughput assay was able to detect simultaneously a panel of informative molecular markers for the presence of CTCs, with time saving and cost-effectiveness. Therefore, a combined panel of these four mRNA markers may not only be a promising tool for the detection of CTCs but also a significant powerful predictor for prognosis, despite normal perioperative serum CEA levels.

Half of the all marker-positive patients developed postoperative relapse, which is significantly higher than the 11.9% of the all marker-negative patients. The false-negative rate (11.9%) of the membrane array method in predicting postoperative relapse, at least in part, might result from CTCs intermittently flowing into the bloodstream of the bowel, the heterogeneous character of the tumor itself, or even the relatively short follow-up period (17, 28, 29). Alternatively, this may be quite reasonable because few carcinoma cells shed into the bloodstream succeed in establishing metastatic disease (30). Using multiple blood sampling (12) or a refined normalization procedure (31) might improve the sensitivity of the membrane array method.

Our results indicated that only 50% of 90 CRC patients with four positive markers developed postoperative relapse. One possible explanation for the high false-positive rate may be that some of our patients received adjuvant chemotherapy (high-risk stage II and stage III CRC), which might affect the postoperative relapse rate. Furthermore, extending the follow-up period may decrease the false-positive rate in predicting relapse. On the other hand, designing new probes to replace the false-positive probes would be useful in improving the false-positive rate of this membrane array assay.

In summary, the constructed membrane array method for the detection of CTCs has been shown to be complementary to the surveillance of CRC patients with normal perioperative serum CEA level. The highly sensitive and high-throughput assay has the clinical potential for early detection of postoperative relapse, with a lead median time of 6 months before the measurement of abnormal CEA levels. However, additional work in a larger patient population by means of serial blood sampling and long-term follow-up is needed to confirm the clinical significance of membrane arrays as adjuvant surveillance.

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